A QUANTITATIVE STUDY OF THE NEUROPATHOLOGY OF THIRTY-TWO SPORADIC AND FAMILIAL CASES OF FRONTOTEMPORAL LOBAR DEGENERATION WITH TDP-43 PROTEINOPATHY (FTLD-TDP)

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Running Head: Familial and sporadic FTLD-TDP

Abstract

Aims: To further characterize the neuropathology of the heterogeneous molecular disorder frontotemporal lobar degeneration (FTLD) with transactive response (TAR) DNA-binding protein of 43kDa (TDP-43) proteinopathy (FTLD-TDP). **Methods:** We quantified the neuronal cytoplasmic inclusions (NCI), glial inclusions (GI), neuronal intranuclear inclusions (NII), dystrophic neurites (DN), surviving neurons, abnormally enlarged neurons (EN), and vacuoles in regions of the frontal and temporal lobe using a phosphorylation independent TDP-43 antibody in thirty-two cases of FTLD-TDP comprising sporadic and familial cases, with associated pathology such as hippocampal sclerosis (HS) or Alzheimer's disease (AD), and four neuropathological subtypes using TDP-43 immunohistochemistry. Analysis of variance (ANOVA) was used to compare differences between the various groups of cases. **Results**: These data from FTLD-TDP cases demonstrate quantitative differences in pathological features between: (1) regions of the frontal and temporal lobe, (2) upper and lower cortex, (3) sporadic and *progranulin* (*GRN)* mutation cases, (4) cases with and without AD or HS, and (5) between assigned subtypes. **Conclusions:** The data confirm that the dentate gyrus is a major site of neuropathology in FTLD-TDP and that most laminae of the cerebral cortex are affected. *GRN* mutation cases are quantitatively different from sporadic cases while cases with associated HS and AD have increased densities of dystrophic neurites (DN) and abnormally enlarged neurons (EN) respectively. There is little correlation between the subjective assessment of subtypes and the more objective quantitative data.

List of abbreviations

AD	Alzheimer's disease
ANOVA	Analysis of variance
CA	Cornu Ammonis
CBD	Corticobasal degeneration
DN	Dystrophic neurites
DG	Dentate gyrus
EN	Abnormally enlarged neurons
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
FTLD-U	Frontotemporal lobar degeneration with ubiquitin positive inclusions
GI	Glial inclusions
GRN	Progranulin (protein)
GRN	*Progranulin* (gene)
HDDD	Hereditary dysphasic disinhibition dementia
IHC	Immunohistochemistry
HS	Hippocampal sclerosis
ITG	Inferior temporal gyrus
MFG	Middle frontal gyrus
MND	Motor neuron disease
NCI	Neuronal cytoplasmic inclusion
NII	Neuronal intranuclear inclusion
NFT	Neurofibrillary tangle
PAX	Progressive apraxia
PHG	Parahippocampal gyrus
PNFA	Progressive non-fluent aphasia
PiD	Pick's disease
SD	Semantic dementia
SP	Senile plaque
STG	Superior temporal gyrus
TDP-43	Transactive response (TAR) DNA-binding protein of 43kD
UBAP1	Ubiquitin-associated binding protein 1
VCP	Valosin-containing protein

Introduction

Frontotemporal lobar degeneration (FTLD) is the second most frequent form of cortical dementia of early-onset after Alzheimer's disease (AD) [1]. The disorder is associated with a heterogeneous group of clinical syndromes including frontotemporal dementia (FTD), FTD with motor neuron disease (FTD-MND), progressive non-fluent aphasia (PNFA), semantic dementia (SD), and progressive apraxia (PAX) [2].

FTLD with transactive response (TAR) DNA-binding protein of 43kDa (TDP-43) proteinopathy (FTLD-TDP), previously called FTLD with ubiquitin immunoreactive inclusions (FTLD-U) [3,4], is characterized by variable neocortical and allocortical atrophy principally affecting the frontal and temporal lobes. In addition, there is neuronal loss, microvacuolation in the superficial cortical laminae, and a reactive astrocytosis [5]. A variety of TDP-43 immunoreactive lesions are present in FTLD-TDP including neuronal cytoplasmic inclusions (NCI), neuronal intranuclear inclusions (NII), dystrophic neurites (DN), and oligodendroglial inclusions (GI).

FTLD-TDP is a heterogeneous molecular disorder [6]. First, there are sporadic and familial forms of the disease; the majority of the latter being caused by mutations in the *progranulin* (*GRN*) gene [7-14]. In addition, cases with *valosin-containing protein (VCP)* gene mutation [15], variants in the *ubiquitin-associated binding protein 1 (UBAP1)* gene [16,17], and common variants at the 7p21 locus have also been shown to be associated with FTLD with TDP-43 inclusions [18]. Second, FTLD can occur in combination with either MND (FTLD-MND), such cases often being associated with a more localized pattern of frontal lobe atrophy [19] or with hippocampal sclerosis (HS) [20], in which there is neuronal loss in the subiculum and sector CA1 of the hippocampus [21]. Some cases within the age range of FTLD-TDP, and especially those of later onset or expressing apoplipoprotein E (APOE) allele ε4, exhibit varying degrees of AD pathology, viz. AD-type senile plaques (SP) and neurofibrillary tangles (NFT). A proportion of cases exhibit a degree of AD pathology greater than expected from normal aging [6]. Third, several attempts have been made to subtype FTLD-TDP [22-24]. Most schemes define four pathological subtypes, based originally on ubiquitin immunohistochemistry (IHC) but extended to

cases of FTLD-TDP, and which utilize the distribution and density of the pathological changes in neocortical regions. The same descriptors have been used to define subtypes but the numbering of each subtype varies between different schemes. Using a composite system proposed by Cairns et al. [24]: type 1 cases (Mackenzie-type 2) are characterized by long DN in superficial cortical laminae with few or no NCI or NII, type 2 (Mackenzie-type 3) by numerous NCI in superficial and deep cortical laminae with infrequent DN and sparse or no NII, type 3 (Mackenzie-type 1) by pathology predominantly affecting the superficial cortical laminae with numerous NCI, DN and varying numbers of NII, and type 4 by numerous NII, and infrequent NCI and DN especially in neocortical areas.

A previous study quantified the pathology in 94 cases of FTLD-TDP obtained from several academic centres [6] and principal components analysis (PCA) was used to compare the degree of similarity and dissimilarity between individual cases. To further characterize the neuropathology of this heterogeneous molecular disorder we quantified, in detail, the pathological changes in various regions of the frontal and temporal lobe in a subset of these cases, viz., thirty-two cases of FTLD-TDP obtained from a single academic centre (Alzheimer's Disease Research Centre, Washington University School of Medicine, St Louis, Missouri, USA). The specific objectives were to compare the densities of the pathological changes between: (1) brain regions, (2) upper and lower cortex, (3) familial and sporadic cases, (4) cases with and without associated HS or AD pathology, and (5) assigned disease subtypes. In this study, analysis of variance (ANOVA) was used to compare the mean densities of histological features between the various groups of cases.

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Materials and Methods

Cases

Thirty-two cases of clinically and neuropathologically well-characterized FTLD-TDP (16 male, 16 female) (see Table 1) were obtained from the Departments of Neurology and Pathology & Immunology, Washington University School of Medicine, St. Louis, MO., USA. All cases exhibited FTLD with neuronal loss, varying degrees of microvacuolation in the superficial cortical laminae, and a reactive astrocytosis

consistent with proposed diagnostic criteria for FTLD-TDP [24]. A variety of TDP-43 immunoreactive lesions were present in these cases including NCI, NII, GI, and DN. Of the 32 cases, 20 were identified as familial (at least one or more first degree relatives affected) and of these, 10 cases were identified as having *GRN* mutations [7,8], one had a *VCP* gene mutation [15], and one case was associated with *UBAP1* [17], a presumptive gene on chromosome 9p21. The majority (N = 7) of the *GRN* cases come from a single hereditary dysphasic disinhibition dementia (HDDD) family (HDDD2) [9] and the remainder (N = 3) from the HDDD1 family. The genetic defects in the remaining familial cases (N = 8) have not been identified to date. None of the cases had coexisting MND (FTLD-MND) [25,26] but six were identified as having associated HS [20]. Identification of cases with additional AD pathology greater than that observed in normal aging [6] was based on Braak's amyloid and NFT scores [27] which are themselves based on the topographical distribution of lesions in circumscribed areas of the limbic system and higher cortical areas. Cases with either an amyloid or NFT score of 4 or greater were considered to have associated AD pathology. Nine such cases were identified, five of which had both amyloid and NFT scores greater than 4 while four cases had only an NFT score greater than 4. Cases were assigned to the four subtypes of FTLD-TDP by an experienced neuropathologist, blind to cases details, based on the composite scheme of Cairns et al. [24]. To provide baseline data for comparison of the densities of surviving neurons, eight control cases, with no neurological or psychiatric histories, within the age range of the FTLD-TDP cases (61-80 years) were also studied.

Histological methods

After death, the consent of the next of kin was obtained for brain removal, following local Ethical Committee procedures (Human Studies Committee, Washington University School of Medicine) and the 1995 Declaration of Helsinki (as modified Edinburgh, 2000). Tissue blocks were taken from the frontal cortex at the level of the genu of the corpus callosum to study the middle frontal gyrus (MFG) and the temporal lobe at the level of the lateral geniculate body to study the inferior temporal gyrus (ITG), parahippocampal gyrus (PHG), CA1/2 sectors of the hippocampus, and dentate gyrus (DG). Tissue was fixed in 10% phosphate buffered formal-saline and embedded in paraffin wax. Following formic acid (95%) pretreatment for 5 minutes,

IHC was performed on 4 - 10µm sections with a rabbit polyclonal antibody (that recognizes TDP-43 (dilution 1:1000; ProteinTech Inc., Chicago, IL). Sections were also stained with haematoxylin.

Both phosphorylation-dependent (pTDP-43) and independent (iTDP-43) antibodies have been used to study the TDP-43 immunoreactive pathological changes in FTLD-TDP [28-31]. Initially we and many other groups used iTDP-43 which immunolabels normal physiological TDP-43 as well as pathological inclusions. This is a particularly useful attribute as this antibody shows clearly that in most neurons there is reduced staining in the nucleus and increased staining of the cytoplasm leading to the hypothesis that the protein is abnormally translocated in disease. However, it is often difficult to distinguish the presence of an abnormal inclusion, or pre-inclusion, in the nucleus, or even the cytoplasm where normal and abnormal staining may admix. The advantage of pTDP-43 antibodies is that they do not immunolabel normal physiological TDP-43 [28,31], especially in the nucleus, thus enabling the TDP-43-immunoreactive lesions to be more clearly visualized and quantified. Nevertheless, studies suggest that the pTDP-43 immunoreactive lesions do not always co-localize with those revealed by iTDP-43 antibodies [30]. To address this question, we compared the densities of iTDP-43- and pTDP-43 immunoreactive lesions in sectors CA1/2 of the hippocampus using the Bland and Altman method [32] to compare the extent of the agreement between the two antibodies.

Morphometric methods

In the MFG, ITG, and PHG of each case, histological features were quantified along strips of tissue (1600 to 3200µm in length), located parallel to the pia mater, using between 32 and 64, 250 x 50µm sample fields arranged contiguously [33]. The sample fields were located both in the upper (approximating to laminae II/III) and lower (approximating to laminae V/VI) cortex; the short edge of the sample field being orientated parallel to the pia mater and aligned with guidelines marked on the slide. In the hippocampus, the features were counted in the cornu ammonis (CA) in sectors CA1 and CA2; the short dimension of the contiguous sample field being aligned with the alveus. NCI have been commonly observed in the DG fascia in FTLD-TDP [3,4,22] and a smaller sample field (50 x 50µm) was aligned with the

upper edge of the granule cell layer. The data were then expressed per 250 x 50μm sample field so that they were directly comparable with those from the other brain regions. The NCI [34,35, GI [36-41], NII [42] and DN [43] were counted in each sample field. Pre-inclusions were not included in the quantification. Surviving neurons were identified as cells containing at least some stained cytoplasm in combination with larger shape and non-spherical outline [44]. By contrast, small spherical or asymmetrical nuclei without cytoplasm, but with the presence of a thicker nuclear membrane and more heterogeneous chromatin, were identified as glial cells. Abnormally enlarged neurons (EN) are a common feature of several neurodegenerative diseases. They are an important aspect of the pathology of tauopathies such as Pick's disease (PiD) [45], corticobasal degeneration (CBD) [46], and argyrophilic grain disease (AGD) [47], and have also been described in Alzheimer's disease (AD) [48]. EN in FTLD-TDP were quantified according to the following criteria: (1) enlarged perikarya lacking NCI, (2) a shrunken nucleus displaced to the periphery of the cell, and (3) a maximum cell diameter of at least three times the diameter of the nucleus [44]. The EN in FTLD-TDP are not tau-immunoreactive but it is not currently known whether they are immunoreactive to beta-crystallin. The number of discrete vacuoles greater than 5μm in diameter was also recorded in each sample field [49,50].

Data analysis

The data were analyzed by analysis of variance (ANOVA) with subsequent comparisons between groups using Fisher's 'protected least significant difference' (PLSD) *post-hoc* procedure (STATISTICA software, Statsoft Inc., 2300 East 14th St, Tulsa, Ok, 74104, USA). First, the densities of each histological feature in the upper cortical laminae of neocortical regions were compared with those in sectors CA1/2 and the DG using a one-way ANOVA. A similar analysis was then carried out but substituting densities in the lower cortical laminae. Second, densities of each histological feature were compared between the upper and lower cortex using two-factor, split-plot ANOVA with brain region as a main-plot factor and cortical laminae as the sub-plot factor. Third, the densities of each histological feature were compared initially between the sporadic cases and all familial cases using unpaired 't' tests, and then between the cases divided into four subgroups, viz., 1) sporadic cases, 2) cases

with *GRN* mutation, 3) cases with other mutations (*VCP, CH9*), and 4) the remaining undetermined familial cases; the groups being compared by a one-way ANOVA. There were no differences between *GRN* mutation cases originating from the HDDD1 and HDDD2 families and these cases were analyzed as a single group. Fourth, densities were compared between cases divided into three subgroups based on the presence of associated pathology, viz., 1) FTLD-TDP with HS, 2) FTLD-TDP with AD pathology, and 3) FTLD-TDP without any apparent associated pathology. In addition, densities were compared between the four subtypes of the disease based on the composite scheme of Cairns et al. [24].

Results

Typical examples if the TDP-43-immunoreactive inclusions are shown in Figs 1a-c. The NCI (Fig 1a) were round or spicular in shape[34,35], while the GI (Fig 1a) morphologically resembled the 'coiled bodies' reported in various tauopathies such as corticobasal degeneration (CBD) [36,37], progressive supranuclear palsy (PSP) [37-39], and argyrophilic grain disease (AGD) [41]. The NII (Fig 1b) were often lenticular, spindle-shaped, or circular in shape [42] and the DN (Fig 1a) were characteristically long and contorted [43]. A typical example of EN in FTLD-TDP is shown in Fig 1c.

A comparison of the densities of TDP-43-immunoreactive inclusions in sectors CA1/2 of the hippocampus in sections immunolabelled with iTDP-43 and pTDP-43 antibodies is shown in Table 2. For the NCI, the overall mean bias was +0.22 (SD = 0.31, CV 0.62). Hence, on average the pTDP-43 antibody revealed +0.22 more NCI per sample field than iTDP-43. For the GI, the mean bias was +0.09 and thus pTDP-43 revealed slightly more GI per plot. For the NII, the mean bias was –0.12 and thus on average pTDP-43 revealed 0.12 fewer lesions than the iTDP-43 antibody. For the DN, the errors were symmetrically distributed approximately around zero (mean bias was –0.04) and therefore very similar densities of DN were revealed by both antibodies.

The densities of the TDP-43-immunoreactive lesions in each brain region, averaged over the thirty-two cases, are shown in Fig 2. Significant differences in density of

NCI were observed between regions when both the upper ($F = 7.43$, $P < 0.001$) and lower ($F = 9.46$, $P < 0.001$) cortex data were included in the analysis. When densities of NCI in the upper cortex were compared with sectors CA1/2 and the DG, densities were greater in the DG compared with all other regions. In addition, the density of NCI was significantly greater in the MFG compared with CA1/2. Results were similar when lower cortex data were substituted in the analysis. Apart from the DG, in which no GI were recorded, there were no significant differences in GI density between regions. Significant differences in the density of NII were observed between regions in the upper ($F = 2.91$, $P < 0.05$) and lower ($F = 3.23$, $P < 0.05$) cortex. When densities in the upper cortex were compared with sectors CA1/2 and the DG, densities were significantly lower in the DG compared with the CA1/2 and the MFG. There were significant differences in the density of DN between regions in the upper cortex only ($F = 3.14$, $P < 0.05$). In addition, the density of DN was significantly lower in the DG compared with all neocortical regions.

The mean densities of the vacuolation, surviving neurons, and EN in each brain region, averaged over cases, are shown in Fig 3. There were significant variations in the density of vacuoles between regions in the upper ($F = 8.34$, $P < 0.001$) and lower ($F = 2.95$, $P < 0.05$) cortex. There were no significant differences in the density of surviving neurons between neocortical gyri. There were significant variations in the density of EN between regions in the upper ($F = 7.18$, $P < 0.001$) and lower ($F = 10.52$, $P < 0.05$) cortex. When densities in the upper cortex were analyzed, the density of the EN was lower in the MFG than the ITG but greater than in the DG. In addition, densities of the EN were greater in CA1/2 compared with the ITG, PHG, and DG. Similar results were obtained when lower cortical data were substituted in the analysis except that the density of vacuoles was significantly lower in the DG but only when compared with the MFG.

The mean densities of surviving neurons in regions of frontal and temporal lobe in the control cases studied are shown in Table 3. Compared with controls, the FTLD-TDP cases collectively show approximately similar reductions in neurons in each of the brain regions examined.

A comparison of the densities of histological features between upper and lower cortex using two-factor ANOVA is shown in Table 4. In all regions, the densities of NCI ($F = 7.41$, $P < 0.01$), DN ($F = 10.27$, $P < 0.01$), and vacuoles ($F = 35.24$, $P < 0.001$) were greater in the upper cortex while the density of NII ($F = 6.35$, $P < 0.01$) was greater in the lower cortex. There were no significant differences in the densities of the GI or surviving neurons between the upper and lower cortex. The distribution of EN was more complex; a significant cortical laminae/region interaction ($F = 5.59$, $P < 0.01$) suggesting that densities of EN were greater in the upper cortex in the MFG but in the lower cortex in the ITG.

A comparison of familial and sporadic FTLD-TDP cases is shown in Table 5. There were no significant differences in the density of histological features between sporadic cases and the familial cases taken as a whole. When cases were divided into four subgroups, however, the density of NCI was greater in the MFG of the *GRN* mutation cases and in cases caused by *VCP* and *CH9*, compared with the sporadic and undetermined familial cases ($F = 3.12$, $P < 0.05$). Moreover, the densities of the vacuoles ($F = 4.03$, $P < 0.05$) and DN ($F = 3.47$, $P < 0.05$) were greater in the *GRN* cases compared with the other familial cases and the sporadic cases. In the DG, there were greater numbers of surviving granule cells in the *GRN* mutation cases compared with the other patient groups ($F = 4.31$, $P < 0.05$).

A comparison of the densities of histological features in FTLD-TDP cases with and without associated HS or AD pathology is shown in Table 6. In the upper cortex of the MTG, there were greater densities of EN ($F = 4.62$, $P < 0.05$) in cases with significant AD pathology. In addition, in the upper cortex of the ITG, there were greater densities of DN ($F = 3.84$, $P < 0.05$) in cases with associated HS and in sectors CA1/2 of the hippocampus, greater densities of NCI in cases with associated AD pathology ($F = 4.05$, $P < 0.05$).

A comparison of the densities of histological features in the FTLD-TDP cases assigned to the four subtypes is shown in Table 7. There were no significant differences between subtypes in the densities of GI or DN in any of the brain regions studied, and in sectors CA1/2 and the DG, no differences in density of any of the pathological features with subtype. There were, however, significant differences in

the density of NCI between subtypes in three regions. Hence, in the upper cortex of the MFG ($F = 3.25$, $P < 0.05$), greater densities of NCI were present in subtype 2 compared with subtype 1, in the upper cortex of the ITG ($F = 4.49$, $P < 0.05$), greater densities in subtypes 2 and 3 compared with subtypes 1 and 4, and in the lower cortex of the ITG ($F = 6.16$, $P < 0.01$), the greatest densities were observed in subtype 2. In addition, there were significant differences in the density of NII between subtypes in three brain regions. In the lower cortex of the MFG ($F = 3.38$, $P < 0.05$) and PHG ($F = 5.57$, $P < 0.01$), and the upper cortex of the ITG ($F = 3.53$, $P < 0.05$), the greatest densities of NII were observed in subtype 4. There were also significant differences in vacuolation between subtypes in 3 regions, with greater densities of vacuoles in subtype 1, and of surviving neurons, EN and subtype each in a single brain region.

Discussion

The objective of this study was to characterize quantitatively the neuropathological differences between brain regions, upper and lower cortex, familial and sporadic cases, cases with and without associated HS or AD pathology, and to further consider the validity of disease subtyping in a group of 32 FTLD-TDP cases obtained from a single academic centre. This particular group of cases contained an unusually high percentage of familial cases and the majority of these were from the HDDD2 family.

Some differences were observed in the densities of lesions in sectors CA1/2 of the hippocampus observed using iTDP-43 and pTDP-43 antibodies. Lesions are easier to observe using the pTDP-43 antibody and therefore the densities of NCI and GI may have been underestimated using the iTDP-43 antibody. By contrast, fewer NII were recorded using the pTDP-43 antibody suggesting that they have been overestimated using iTDP-43. However, whether or not all TDP-43 immunoreactive lesions are phosphorylated and the significance of phosphorylation in the development of TDP-43 pathology in FTLD-TDP remains to be established [29].

In the FTLD-TDP cases as a whole, there were quantitative differences in the density of several histological features between brain regions. First, the greatest densities of NCI were observed in the DG, densities often being significantly lower in the adjacent sectors CA1/2 of the hippocampus and the PHG; thus confirming the DG as

a significant site of temporal lobe pathology in FTLD-TDP [3,4,22]. By contrast, densities of the NII were usually greater in the frontal and temporal neocortex and in sectors CA1/2 of the hippocampus while DN were most abundant in the neocortex; densities of both lesions being low in the DG. The GI exhibited the lowest densities of the TDP-43-immunoreactive inclusions recorded and apart from the DG, exhibited no apparent regional preference. The density of EN was low in the FTLD-TDP cases generally and significantly lower than reported in various tauopathies such as corticobasal degeneration (CBD) [37], and Pick's disease (PiD) [51]. By contrast, vacuolation [49,50] was a consistent feature of neocortical regions, especially of the upper cortex, densities being significantly lower in the lower cortex and in the hippocampus.

Differences in the density of various pathological features were also observed between upper and lower cortical laminae. The NCI and DN, for example, were present at higher densities in the upper cortex and NII in the lower cortex suggesting that degeneration occurs across the cortex in FTLD-TDP; the NCI/DN and NII affecting different populations of neurons. The relative distributions of the vacuoles and surviving neurons suggest it is the upper cortex that is affected to the greatest extent. In addition, there were no significant differences in the density of surviving neurons in the upper and lower cortex. In association cortex of normal brain, the distribution of the larger pyramidal neurons across the cortex is often double-peaked (bimodal); the two peaks of density corresponding spatially to laminae II/III and V/VI with the larger of the two density peaks in the upper cortex [37]. Hence, the similarity in the density of neurons in the upper and lower cortex in our cases is also consistent with a greater neuronal loss affecting the upper laminae in FTLD-TDP.

Differences in density of various pathological features were observed between familial and sporadic cases of FTLD-TDP. The majority of the familial cases in the present study had *GRN* mutations from the HDDD2 family [9]. These *GRN* cases had consistently higher densities of NCI, DN, and vacuoles in the MFG compared with the sporadic cases. Previous studies report that DN were more frequent in the frontal cortex and less frequent in the DG in *GRN* cases consistent with our data [43,52]. In addition, cases lacking *GRN* mutations may have a less severe pathology affecting the neocortex and striatum while NII are usually absent or infrequent [10]. The density of

NII, however, was statistically similar in our sporadic and familial cases but greater neuronal preservation was suggested within the DG in the *GRN* cases.

In FTLD-TDP cases with HS, significantly higher densities of DN were recorded in the ITG, while cases with significant AD pathology had higher densities of EN in the MTG and NCI in sectors CA1/2 of the hippocampus. HS is usually associated with enhanced neuronal loss in the subiculum and sector CA1 of the hippocampus [21] but there were no differences in the density of surviving neurons in these regions in the present cases with or without HS. Moreover, the density of EN was low in our FTLD-TDP cases [6] but the data did suggest that increased densities of EN could be associated with the presence of enhanced AD pathology. The increase in the density of TDP-43-immunoreactive NCI in sectors CA1/2 of the hippocampus in cases with associated AD pathology is also of interest as large numbers of NFT occur in these sectors in 'pure' AD [53]. Hence, in FTLD-TDP with associated AD pathology, a proportion of the NCI in sectors CA1/2 could be AD-NFT but with the inclusions having acquired TDP-43 immunoreactivity [54].

Several authors have attempted to divide FTLD-TDP cases into pathological 'subtypes' [22-24] and many continue to find the classification sufficiently robust to differentiate cases effectively. In the multicentre study of Cairns et al. [5], for example, the majority of the *GRN* mutation cases were assigned to subtype 3 and VCP to subtype 4. In the present study, in which subtypes were assigned blind to the details of the cases, although some *GRN* mutation cases were assigned to subtype 3, subtypes 1, 2 and 4 were also represented. Quantitative differences were observed between the cases assigned to the various subtypes and especially greater densities of NCI in subtype 2 and NII in subtype 4, but these are subtle quantitative differences rather than the large qualitative variations necessary for ease of subtyping. In addition, quantitative differences between subtypes were not consistently present in different brain regions. The previous quantitative study of 94 FTLD-TDP cases from several centres, and which included the present cases, showed that apart from the subtype 1 cases which did appear to more distinct, the FTLD-TDP cases formed a 'continuum' and there was broad overlap between the subtypes, especially between subtypes 2 and 3. The original classification schemes were based on ubiquitin IHC, on the relative numbers of lesions, and on their laminar distribution. Hence, although

the classification scheme has been extended to include TDP-43 IHC, quantitative pathological information based on TDP-43 IHC may have added complexity or noise to the data. Overall there remains little correlation between the subjective assessment of subtypes assigned blind to the details of the cases and the more objective quantitative data. This discrepancy may depend on (1) the cases studied may have been especially difficult to assign to subtype, (2) a restricted number of brain regions were sampled and hence, a better correlation might have been obtained if the quantitative study was extended to the motor cortex, basal ganglia, amygdala, midbrain, and medulla, (3) the skill and experience of neuropathologists involved in subtyping, and (4) the use of the iTDP-43 antibody. To clarify this question further, it would be useful to conduct a large-scale study of FTLD-TDP cases to assess inter-rater reliability in assigning the four subtypes.

In conclusion, significant variations in TDP-43 pathology were observed between regions in the frontal and temporal lobe; the greatest densities of NCI being recorded in the DG, NII in the lower cortex and in sectors CA1/2 of the hippocampus, and DN in the upper cortex. Cases caused by *GRN* mutations may be associated with greater densities of NCI, DN, and vacuoles in some areas while cases with associated HS or with significant AD pathology also show differences in density. There are some differences in density of NCI and NII between disease subtypes but these do not provide an objective basis for subtyping. The classification of FTLD-TDP cases as a whole is under review and new variations and subtypes of the disease are likely to be proposed and therefore, detailed quantitative data and analysis may be of value in elucidating this complex molecular pathology.

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Table 1. Summary of demographic features (M/F = Male/female, Dur = Disease duration, Death = Age at death), gross brain weight (BW), familial/sporadic status (Fm/S) (*GRN* = FTLD-TDP with *progranulin* gene mutations, (1) indicates *HDDD1* family and (2) *HDDD2* family; *VCP* = FTLD-TDP with valosin-containing protein, *CH9* = FTLD-TDP linked to a putative gene on chromosome 9), associated pathology (AP) (N = no associated pathology, HS = hippocampal sclerosis, AD = significant Alzheimer's disease pathology), pathological subtype (ST) (ND = subtype could not be determined, 1/2 = case intermediate between subtype 1 and 2) of the 32 cases of

frontotemporal lobar degeneration with TDP proteinopathy (FTLD-TDP), (- data not available).

Case	M/F	Onset	Dur	Death	BW	Fm/S	AP	ST
1.	M	57	8	65	960	S	N	2
2.	F	72	12	84	900	S	HS	2
3.	F	61	16	77	950	S	N	3
4.	F	68	6	74	975	*GRN (2)*	N	4
5.	M	52	13	65	1300	Fm	AD	1
6.	M	66	16	82	-	S	HS	3
7.	F	69	15	84	970	*GRN (1)*	AD	3
8.	M	60	6	66	-	*GRN (1)*	AD	1
9.	F	65	12	77	810	*GRN (1)*	N	1
10.	M	52	15	67	960	*GRN (2)*	N	4
11.	M	74	6	80	1270	*GRN (2)*	AD	4
12.	F	-	-	67	990	S	N	2
13.	F	59	9	68	650	Fm	N	2
14.	M	74	1	75	1360	S	N	1
15.	M	60	11	71	1450	Fm	HS	4
16.	M	43	7	50	1060	Fm	N	3
17.	M	55	11	66	1005	S	HS	2
18.	F	63	3	66	950	S	N	1
19.	F	58	9	67	880	*GRN (2)*	HS	2
20.	F	64	19	83	720	Fm	N	1/2
21.	F	69	4	71	1070	S	N	4
22.	M	38	9	47	1185	*VCP*	N	4
23.	F	-	-	73	720	S	N	1
24.	M	50	18	68	1170	S	HS	1
25.	M	58	8	66	1080	Fm	AD	4
26.	F	65	13	78	960	Fm	N	ND
27.	M	57	6	63	1080	*GRN (2)*	N	3
28.	M	51	11	62	880	*CH9*	AD	2
29.	F	71	13	84	960	S	AD	1

30.	F	73	9	82	-	*GRN (2)*	AD	2
31.	F	58	8	66	-	Fm	N	4
32.	M	71	8	79	1150	*GRN (2)*	AD	3

Table 2. Summary of the degree of agreement between the densities of the TDP-43 immunoreactive lesions (NCI = Neuronal cytoplasmic inclusions, GI = Glial inclusions, NII = Neuronal intranuclear inclusions, DN = Dystrophic neurites) in the CA sectors of the hippocampus using phosphorylation-dependent (pTDP-43) and phosphorylation-independent (iTDP-43) antibodies using the Bland and Altman graphical method (SD = Standard deviation, CV = Coefficient of variation).

Lesion	Bias	SD	CV	Limits of agreement
NCI	+0.22	0.31	0.62	-0.39 to 0.84
GI	+0.09	0.24	0.48	-0.39 to 0.57
NII	-0.12	0.21	0.42	-0.54 to 0.29
DN	-0.04	0.34	0.66	-0.70 to 0.63

Table 3. Mean densities of surviving neurons (250 x 50μm field) in regions of the frontal and temporal lobe (MFG = Middle frontal gyrus, ITG = Inferior temporal gyrus, PHG = Parahippocampal gyrus, CA1/2 = Sectors CA1/2 of the hippocampus, DG = Dentate gyrus) in control cases (SE = Standard error of the mean)

	Brain region				
	MFG	ITG	PHG	CA1/2	DG
Mean	9.55	8.33	8.14	4.89	18.18
SD	1.08	2.07	1.30	1.06	3.09

Table 4. Summary of the results of two-factor analyses of variance (ANOVA) of the densities of histological features (NCI = neuronal cytoplasmic inclusions, GI = oligodendroglial inclusions, NII = neuronal intranuclear inclusions (NII), EN = abnormally enlarged neurons, SN = surviving neurons, V = vacuolation, DN = dystrophic neurites) in the upper and lower cortex in cases of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP) (Data are 'F' ratios from two factor, split-plot analysis of variance (ANOVA) * P < 0.05; ** P < 0.01; *** P < 0.001).

	Factorial effect		
Histological feature	Brain region	Upper/Lower cortex	Interaction
NCI	F = 0.62	F = 7.41**	F = 0.11
GI	F = 1.50	F = 1.82	F = 0.12
NII	F = 0.56	F = 6.35**	F = 0.20
EN	F = 0.54	F = 1.15	F = 5.59**
SN	F = 2.68	F = 3.79	F = 0.38
V	F = 1.12	F = 35.24***	F = 1.56
DN	F = 0.27	F = 10.27**	F = 0.85

Table 5. Comparison of the densities (per 50 x 250μm field, standard errors in parentheses) of the pathological changes (NCI = neuronal cytoplasmic inclusions, DN = dystrophic neurites) between familial (*GRN* = cases caused by *progranulin* mutations) and sporadic cases of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Data show brain areas (MFG = Middle frontal gyrus, DG = dentate gyrus) and pathological features that exhibit a significant difference between groups.

			FTLD-TDP group		
Brain region	Histological feature	Sporadic cases	*GRN* mutation cases	Other mutation cases	Other familial cases
MFG (upper cortex)	NCI	0.12 (0.05)	0.36 (0.06)	0.22 (0.13)	0.14 (0.06)
	Vacuoles	8.87 (1.82)	16.75 (2.02)	5.38 (4.26)	9.26 (1.91)
	DN	0.29 (0.15)	0.78 (0.17)	0	0.08 (0.16)
DG	Surviving neurons	13.42 (0.86)	17.49 (0.09)	14.12 (2.02)	13.4 (0.91)

ANOVA (1-way): MFG, NCI $F = 3.12$ ($P < 0.05$); Vacuoles $F = 4.03$ ($P < 0.05$); DN, $F = 3.47$ ($P < 0.05$); DG, Surviving neurons $F = 4.31$ ($P < 0.01$).

Table 6. Comparison of the densities (per 50 x 250μm field, standard errors in parentheses) of the pathological changes (NCI = neuronal cytoplasmic inclusions, DN = dystrophic neurites, EN = abnormally enlarged neurons) between cases with and without associated pathologies (AP) (AD = with significant Alzheimer's disease pathology, HS = Hippocampal sclerosis) in cases of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Data show brain regions (MFG = Middle frontal gyrus, ITG = Inferior temporal gyrus) and pathological features that exhibit a significant difference between groups.

		Patient group		
Brain Region	Pathological feature	No AP	FTLD/AD	FLTD/HS
MFG (upper cortex)	EN	0.08 (0.03)	0.24 (0.04)	0.07 (0.05)
ITG (upper cortex)	DN	0.33 (0.14)	0.17 (0.19)	0.99 (0.23)
Sectors CA1/2	NCI	0.03 (0.02)	0.13 (0.03)	0.03 (0.04)

ANOVA (1-way): MFG, EN: $F = 4.62$ ($P < 0.05$); ITG, DN: $F = 3.84$ ($P < 0.05$); DN: $F = 3.47$ ($P < 0.05$), CA1/2, NCI: $F = 4.05$ ($P < 0.05$).

Table 7. Comparison of the densities (per 50 x 250μm field, standard errors in parentheses) of the pathological changes (NCI = neuronal cytoplasmic inclusions, NII = neuronal intranuclear inclusions, SN = surviving neurons, EN = abnormally enlarged neurons) between the four subtypes of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP) based on the composite scheme of Cairns et al (2007b). Data show brain regions (MFG = Middle frontal gyrus, ITG = Inferior temporal gyrus, PHG = parahippocampal gyrus) and pathological features that exhibit a significant difference between groups.

		Subtype			
Brain region	Pathological feature	1	2	3	4
MFG (upper cortex)	NCI	0.06 (0.04)	0.37 (0.06)	0.28 (0.08)	0.15 (0.07)
MFG (lower cortex)	NII	0.19 (0.06)	0.18 (0.06)	0.13 (0.06)	0.39 (0.06)
ITG (upper cortex)	NCI	0.08 (0.05)	0.29 (0.05)	0.28 (0.06)	0.10 (0.05)
	NII	0.04 (0.02)	0.08 (0.03)	0.09 (0.06)	0.32 (0.07)
	SN	5.03 (0.71)	6.45 (0.73)	7.82 (0.93)	3.67 (0.71)
	Vacuoles	10.53 (1.24)	8.46 (1.24)	10.11 (1.57)	5.30 (1.24)
ITG (lower Cortex	NCI	0.06 (0.03)	0.31 (0.05)	0.02 (0.01)	0.04 (0.02)
	Vacuoles	10.26 (0.70)	2.30 (0.69)	6.21 (0.77)	5.45 (0.69)
PHG (upper cortex)	Vacuoles	16.31 (1.78)	6.36 (1.75)	10.34 (2.27)	6.81 (1.80)
PHG (lower cortex)	NII	0.05 (0.03)	0.20 (0.06)	0.26 (0.08)	0.42 (0.06)
Sectors CA1/2	EN	0.15 (0.04)	0.05 (0.04)	0.22 (0.04)	0.07 (0.04)

ANOVA (1-way): MFG-upper, NCI: $F = 3.35$ ($P < 0.05$) Subtype 2 > 1 and 2 > 4; MFG-lower NII $F = 3.40$ ($P < 0.05$), Subtype 4 >1,2,3; ITG-U, NCI: $F = 4.51$ ($P < 0.05$), Subtype 2,3 > 1,4; NII $F = 3.53$ ($P < 0.05$), Subtype 4 >1,2,3; SN $F = 4.35$ ($P < 0.05$), Subtype 2,3 > 1,4; Vacuoles $F = 3.46$ ($P< 0.05$), Subtype 1,3 > 4; ITG-lower NCI $F = 7.21$ ($P < 0.01$), subtype 2 > 1,3,4; Vacuoles $F = 5.46$ ($P < 0.01$), Subtype 1

> 2,4; PHG-upper Vacuoles F = 5.01 ($P < 0.01$), Subtype 1 > 2,4; PHG-lower NII F = 5.57 ($P < 0.01$), Subtype 4 > 1,2; CA1/2 EN F = 3.77 ($P < 0.05$), Subtype 3 > 2,4.

Legends to figures

Fig 1a. Frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). TDP-43 immunoreactive lesions in the frontal cortex showing neuronal cytoplasmic inclusion (NCI) (arrow), dystrophic neurite (DN) (double arrow), and glial inclusion (GI) (arrow head) (TDP-43 immunohistochemistry, bar = 50μm).

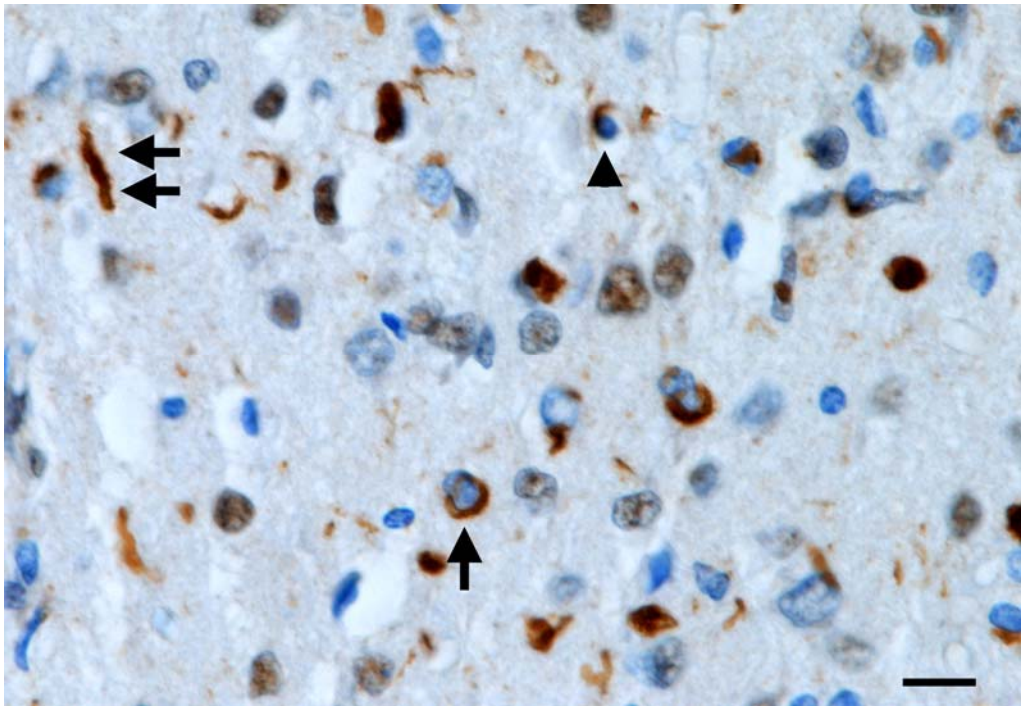

Fig 1b. Frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Neuronal intranuclear inclusion (NII) in lamina III of the superior temporal gyrus (TDP-43 immunohistochemistry, bar = 10μm).

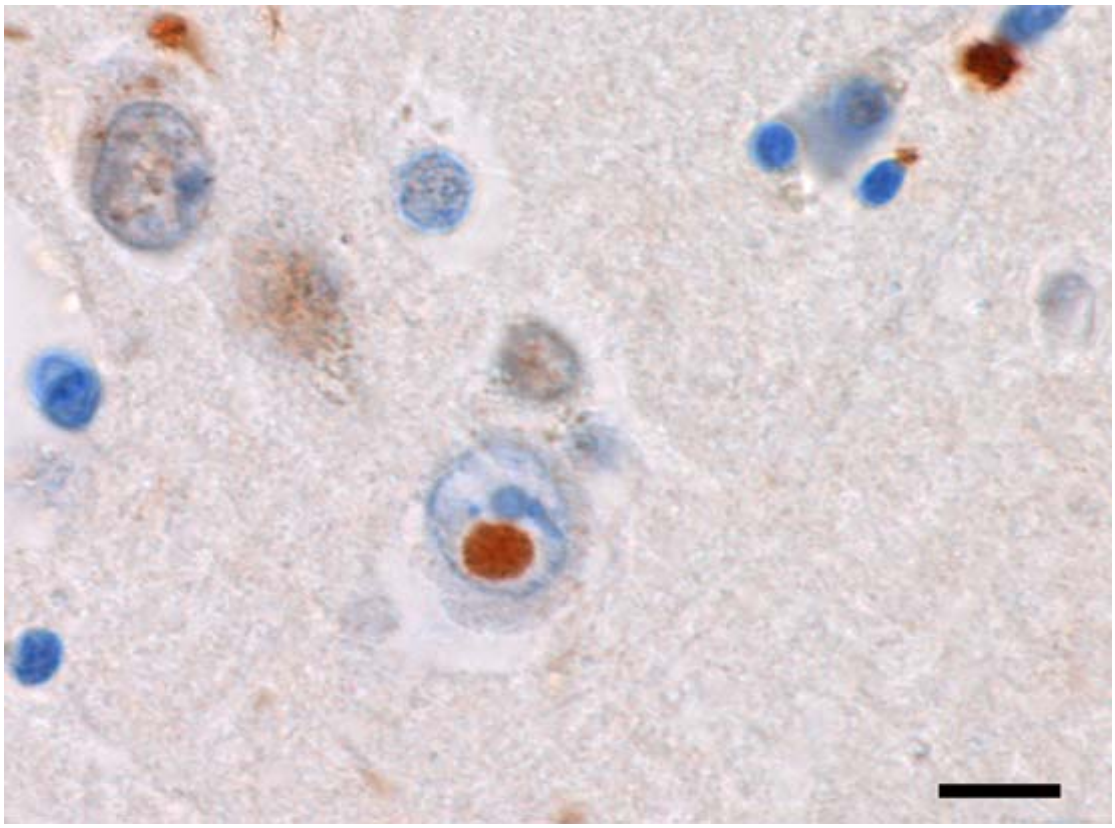

Fig 1c. Frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Abnormally enlarged neuron (EN) (arrows) in lamina III of the superior temporal gyrus (H/E, bar = 20μm).

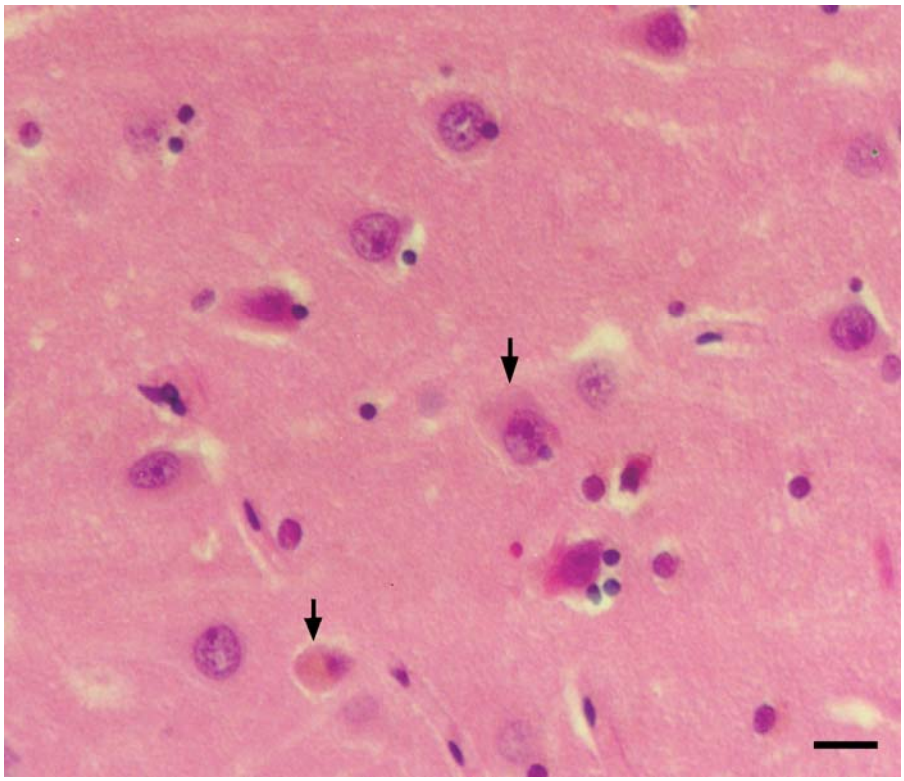

Fig 2. Mean densities (error bars indicate standard errors) of the TDP-43 immunoreactive pathological features (NCI = neuronal cytoplasmic inclusions, GI = oligodendroglial inclusions, NII = neuronal intranuclear inclusions (NII), DN = dystrophic neurites,) in the frontal and temporal lobe (MFG = Middle frontal gyrus, ITG = Inferior temporal gyrus, PHG = Parahippocampal gyrus, CA1/2 = Sectors CA/2 of the hippocampus, DG = Dentate gyrus), U = Upper cortex, L = lower cortex) of cases of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Analysis of variance (1-way): 1) Comparing upper cortex, CA1/2, and DG data: NCI, $F = 7.43$ ($P < 0.001$); GI, $F = 3.71$ ($P < 0.01$); NII, $F = 2.91$ ($P < 0.05$); DN, $F = 3.14$ ($P < 0.05$); 2) Comparing lower cortex, CA1/2, and DG data: NCI, $F = 9.46$ ($P < 0.001$); GI, $F = 3.54$ ($P < 0.01$); NII, $F = 3.23$ ($P < 0.05$); DN $F = 2.08$ ($P > 0.05$).

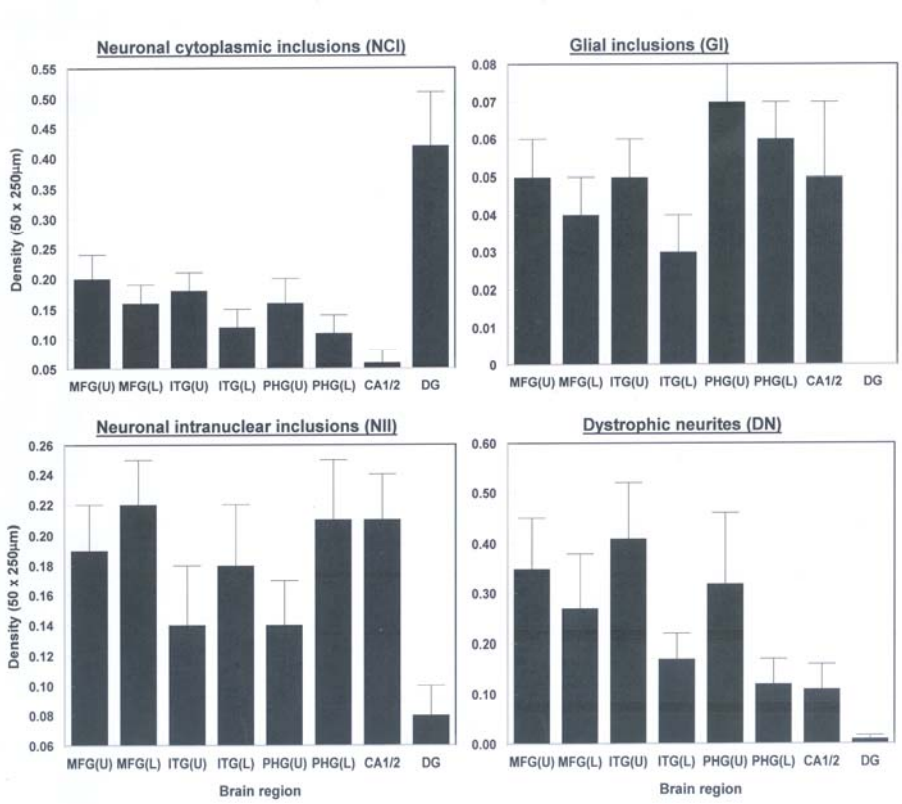

Fig 3. Mean densities (error bars indicate standard errors) of the vacuolation, surviving neurons, and enlarged neurons (EN) in the frontal and temporal lobe (MFG = Middle frontal gyrus, ITG = Inferior temporal gyrus, PHG = Parahippocampal gyrus, CA1/2 = Sectors CA/2 of the hippocampus, DG = Dentate gyrus), U = Upper cortex, L = lower cortex) of cases of frontotemporal lobar degeneration with TDP-43 proteinopathy (FTLD-TDP). Analysis of variance (1-way): 1) Comparing upper cortex, CA1/2, and DG data, Vacuoles $F = 8.34$ ($P < 0.001$), Surviving neurons = 131.97 ($P < 0.001$), EN $F = 7.18$ ($P < 0.001$); 2) Comparing lower cortex, CA1/2, DG data: Vacuoles $F = 2.95$ ($P < 0.05$), Surviving neurons $F = 166.89$ ($P < 0.001$), EN $F = 10.52$ ($P < 0.001$).